PROTEIN QUALITY CONTROL AT THE PLASMA MEMBRANE:

INSIGHT INTO THE ROLE OF LIPID MICRODOMAINS

by

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ABSTRACT

Protein homeostasis refers to the cellular processes that regulate the folding, unfolding, misfolding, trafficking and degradation of proteins. These cellular processes include the protein expression pathway, adaptive profolding mechanisms and protective degradative pathways. Plasma membrane proteins are located at the interface between the internal and external environment of the cell. They are essential for cellular integrity. Molecules that cannot diffuse across the plasma membrane have to pass through transport proteins that span the length of the plasma membrane. Damage to these transporters can cause unrestricted entry and exit of molecules, an outcome that eventually leads to cellular demise. The levels of these transporters are hence closely regulated by the cell through evolutionarily conserved mechanisms. In the event of an insult to the transporter, the profolding pathways are initially activated. If these fail, the protein is downregulated through the degradative quality control machinery in order to prevent aggregation and persistent defect in function. In *Saccharomyces cerevisiae, the* downregulation of the uracil transporter Fur4 is mediated through ubiquitin-dependent endocytosis and trafficking to the vacuole for degradation. The ubiquitin ligase Rsp5 is responsible for identifying an unfolded Fur4 and catalyzes its ubiquitination at lysine residues. It remains an open question how the soluble Rsp5 is recruited to the plasma membrane. It is also not clear how lipid microdomains impact this ubiquitination event.

Previous studies have described the LID-degron system as a mechanism involved in Fur4 dowregulation. Utilizing the LOV2 photosystem and artificial degrons, I have

separated conformational changes within the transporter from degron exposure. This has allowed us to independently expose the degron and monitor the dynamics of Rsp5 recruitment. Our results suggest that a transmembrane adaptor is involved in Rsp5 recruitment and that plasma membrane microdomain dynamics ensure that ubiquitination occurs after lateral translocation.

Dedicated to my loving wife, Elizabeth. Her continued support made this project a possibility.

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LIST OF ABBREVIATIONS

APC ..... Amino Acid-Polyamine-Organocation

ART ..... Arrestin-Related Trafficking Adaptors

ER ..... Endoplasmic Reticulum

ESCRT ..... Endosomal Sorting Complexes Required for Transport

ILV.....Intraluminal Vesicles

LID.....Loop Interaction Domain

MVB .....Multivesicular Body

PI-3P .....Phosphatidylinositol 3-Phosphate

LOV.....Light/Oxygen/Voltage

VPS .....Vacuolar Protein Sorting

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CHAPTER 1

INTRODUCTION

1.1 Proteostasis

Protein homeostasis entails the evolutionarily conserved cellular processes involved in maintaining the proteome in a functional state. It is responsible for the folding, unfolding, misfolding, trafficking and degradation of proteins (Balch et al., 2008). These tasks can be daunting because of the marginal stability of proteins in vivo, as well as the often unfavourable environmental and metabolic conditions frequently encountered. These conditions include oxidative stresses, starvation and overwhelming substrate concentration (Seron et al., 1999; Blondel et al., 2004; Jones et al., 2012).

Yet, it is crucial to ensure a continued folded conformation since proteins perform the majority of cellular functions. At the plasma membrane, for instance, permanent unfolding of a few integral membrane proteins can cause loss of cell integrity, while unfolded soluble proteins can aggregate and induce proteotoxicity. These defective protein states are pathogenetic in disease states like cystic fibrosis and neurodegenerative diseases (Benharouga et al., 2001; Ross et al., 2004; Chiti et al., 2006; Ciryam et al., 2013). Hence, the cell has developed efficient proteostatic machinery that exists at the plasma membrane as well as in subcellular organelles like the mitochondria (Haynes and Ron, 2010), in autophagic processes and in the endocytosis-exocytosis system (Sridhar et al., 2012; Hutt and Balch, 2013). This process is coordinated by arrays of chaperones and

factors that number in the thousands in the mammalian cell (Kim et al., 2013).

The proteostatic system is best described as consisting of the protein expression pathway that regulates protein synthesis, adaptive profolding mechanisms called into play in the face of stressors, as well as protective degradative pathways that function in clearing unfolded proteins. The profolding mechanisms include the unfolded protein response (UPR) (Walter and Ron, 2011), heat-shock response (Morimoto, 2011), antioxidant and redox signaling (ARS) (Margittai and Sitia, 2011) and the mitochondrial response (mitoUPR) (Haynes and Ron, 2010).

On the other hand, if the above fails, the protective pathways are activated as degradative quality control machinery that prevents aggregation and persistent defects in function. These include the autophagy-lysosome pathway and the ubiquitin-proteosome pathway (Finley, 2009; Metcalf et. al., 2012). The rest of this paper will discuss quality control at the plasma membrane as it involves nutrient transporters in yeast because little is known about the mechanisms of protein quality control at the plasma membrane. For example, nutrient transporters have small cytoplasmic domains that render them largely inaccessible to molecular chaperones, the major quality control system of cytoplasmic proteins. In this study, I have chosen *Sacharomyces cerevisiae* due to its genetic tractability.

1.2 Quality control pathway for transmembrane proteins in yeast

The degradation of unfolded transmembrane proteins in yeast occurs through the Multivesicular Body Pathway (MVB). This is a ubiquitin-dependent endocytotic pathway (Kolling and Hollenberg, 1994; Hicke and Riezman, 1996; Egner et al., 1996; Galan et al., 1996; Jenness et al., 1997) starting with the ubiquitination of

damaged plasma membrane proteins at lysine residues. Ubiquitination is a two-step conjugation reaction in which an isopeptidic bond is formed between the C-terminal carboxyl group of ubiquitin (a 76-amino acid peptide) and the epsilon-amino group of a lysine residue in the target protein (Komander, 2009; Ye and Rape, 2009). This reaction is catalyzed sequentially by three enzymes; E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) (Deshaies and Joazeiro, 2009; Nagy and Dikic, 2010; Wenzel et al., 2011). Rsp5 is the predominant E3 (ubiquitin ligase) of the yeast endocytic system, and it plays a key role in the trafficking of damaged transmembrane proteins, a process in which ubiquitin serves as the sorting signal. This signal can be either monoubiquitin or polyubiquitin, however, multi-monoubiquitin is more common. The consensus in the field is that while K-48 linked polyubiquitination predominates in the sorting of soluble proteins to the proteasome, K-63 monoubiquitination and K-63 linked polyubiquitination is the major signal for the trafficking of plasma membrane proteins (Hoeller and Dikic, 2010; Lauwers et al., 2010). Ubiquitination can be reversed by the action of deubiquitinating enzymes (DUBs), which catalyze the proteolytic cleavage of the isopeptidic bonds between ubiquitin and lysine (Komander et al., 2009). This reversibility allows ubiquitination to function as a key switch in the sorting pathway, wherein it can be conjugated when a protein is unfolded and deconjugated when the protein refolds.

How the ubiquitin-tagged defective membrane proteins are recognized and retrieved into endocytic vesicles remains unclear. However, it is widely hypothesized that there are proteins with ubiquitin-binding domains that bind these proteins and mediate their transfer into vesicles through clathrin-based mechanisms. Examples of such proteins

are Ent1 and Ent2, which are known to bind to ubiquitin via ubiquitin-interacting motif domains at one end, and interact with clathrin, the AP-2 adaptor on the other end (Sigismund et al., 2005). In the same vein, Ede1 binds through its N-terminal EH domains to endocytic proteins on one hand and ubiquitinated proteins on the other hand via its C-terminal ubiquitin-interacting motifs (Polo et al., 2002). Other similar proteins containing ubiquitin-binding domains and implicated in endocytosis of plasma membrane proteins include Sla1 and Lsb5 (Costa et al., 2005; Stamenova et al., 2007). However, the precise mechanistic interactions of these proteins with the endocytotic machinery are not known.

From the plasma membrane, endocytosed proteins (Figure 1.1, step 1) are ferried in vesicles to the early endosome to which they fuse. It is here that critical sorting decisions are made (Piper et al., 2014). Proteins that refold successfully are deubiquitinated by deubiquitinating enzymes, recruited to the cytosolic surface of the early endosome. The deubiquitinated proteins are then recycled back to the plasma membrane (Figure 1.1, step 2). This recycling appears to be a common occurrence as studies of the cystic fibrosis transconductance regulator (CFTR; mutated in cystic fibrosis patients) have shown up to 70% recycling rates (Swiatecka-Urban et al., 2005). On the other hand, if refolding is unsuccessful, Rsp5 will catalyze reubiquitination of the protein and this can then serve as a signal for progression (Figure 1.1, step 3) to the multivesicular body. The multivesicular body (Figure 1.1, step 4) is formed when the limiting membrane of the endosome invaginates to form an intraluminal vesicle containing the unfolded protein, while the ubiquitin is removed and recycled (Luhtala and Odorizzi, 2004). This process is unique because the vesicle formation is directed into the lumen of the endosome, which is topologically distinct from other vesicle formation

events in the cell (e.g. clathrin-mediated endocytosis). MVB vesicle formation is mediated by a network of protein complexes collectively called Endosomal Sorting Complexes Required for Transport (ESCRTs) (Shields and Piper, 2011; Babst M, 2011). The ESCRTs consists of: ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and the Vps4 AAA-ATPase complex (Prag et al., 2007; Ren et al., 2009; Shields et al., 2009; Hurley, 2010; Wollert and Hurley, 2010 ; Mayers, 2011; Ren and Hurley, 2011; Shields and Piper, 2011). The process starts with the recruitment of ESCRT-0 to the endosomal membrane where it binds to the head group of phosphatidylinositol 3-phosphate (PI-3P). At this location, the recognition of ubiquitinated membrane proteins by ESCRT-0 leads to the initiation of cargo sorting and subsequent recruitment of ESCRT-I which also helps in sorting the ubiquitinated protein (Katzmann and Babst, 2001; Henne et al., 2010). ESCRT-I then recruits ESCRT-II whose function is to initiate the formation of ESCRT-III through a polymerization reaction. Together, ESCRT-II and III concentrate cargo proteins on the endosomal membrane and cause membrane deformation leading to formation of intraluminal vesicle (Schmidt and Teis, 2012). This occurs after the proteins have been deubiquitinated by the deubiquitinating enzyme Doa4. Finally, the ESCRT complex diassembles and the VPS-4 complex catalyses fission at the neck of the intraluminally directed vesicle (Henne et al., 2010). The ESCRT protein complexes and their subunits are listed in Table 1.1

The mature MVB fuses with the vacuole (lysosome in mammalian cells) and delivers its content into the acidic milieu of the vacuole for degradation (Figure 1.1, step 5). The amino acid by-products of this degradation are then subsequently pumped into the cytosol where they are salvaged and reused for de novo protein synthesis.

1.3 Fur4 as a model nutrient transporter degraded in the yeast MVB pathway

To gain further insights into the quality control of transmembrane proteins in this project, I have utilized Fur4, the high-affinity nutrient transporter in yeast. Nutrient transporters function as a port between the intracellular and extracellular milieu, hence proper functionality is crucial for cellular integrity. This dictates the need for efficient quality control systems to quickly detect unfolding and initiate downregulation of defective proteins before cellular damage occurs.

Fur4 is a 633-residue uracil permease that belongs to the nucleobase:cation symporter-1 (NCS1) family of transporters (Jund et al., 1988). The NCS1 family further belong to the larger Amino Acid-Polyamine-Organocation *(*APC*)* Superfamily. Members of this family act in purine and pyrimidine uptake through symport with H+ or Na+. The NCS1 family includes over 1000 evolutionarily conserved proteins across bacteria, yeast, fungi and plants. Other members of the family include the bacterial benzyl-hydantoin transporter, Mhp1, and yeast thiamine permease, Thi10 (Belenky et al., 2008). They contain twelve transmembrane domains with N- and C-terminal hydrophilic sequences. Most of the functional attributes of Fur4 were deduced from comparisons made with its bacterial homologue, Mhp1 from *Microbacterium liquefaciens*. The structure of Mhp1, resolved to 2.85 Å, consists of 12 transmembrane helices. Structural information, gleaned from the outward open and substrate bound occluded states, shows that the outward-facing cavity closes upon binding of substrate. The alternating access model for membrane transport by NCS1 family proteins was based on structural information from Mhp1 showing that the reciprocal opening and closing of the inward and outward facing cavities is synchronized by the 3rd and 8th inverted repeat helices (Oleg, 1966; Jund et

al., 1988; Shimamura et al., 2010). The model was further supported by conclusions, drawn from comparisons made with the leucine transporter LeuTaa and the galactose transporter vSGLT, that the outward and inward facing cavities are arranged symmetrically on opposite sides of the membrane. The alternating access model starts with the outward-facing open conformation (Figure 1.2 step 1), in which the substrate and ions can enter the binding sites, located near the center of the protein, from the extracellular space. A conformational change then occurs that seals off ion and solute in this occluded outward facing state (Figure 1.2 step 2). The protein then switches to the inward-facing occluded conformation (Figure 1.2 step 3), and subsequently to the inward-facing open state (Figure 1.2 step 4), in which the binding sites become continuous with the intracellular compartment causing a release of ion and solute. Following the discharge of its contents, the transporter then reverts back to its ground state (Figure 1.2 step 5), which is the outward-facing open conformation (Oleg, 1966; Jund et al., 1988; Shimamura et al., 2010).

1.4. Mechanisms of plasma membrane protein downregulation

The downregulation of transmembrane nutrient transporters is usually initiated by protein-specific events or the starvation response pathway (Lang et al., 2014). Protein-specific events refer to either high substrate concentration or protein unfolding. Plasma membrane quality control focuses on the degradation of unfolded proteins. Two mechanisms underlying plasma membrane quality control have been identified: chaperone-dependent quality control and the quality control mediated by the LID-degron system. These mechanisms aim to explain how the unfolded states of proteins are recognized and retrieved from the plasma membrane, along with the

factors required for this process.

1.4.1 Chaperone-dependent quality control mechanism

This quality control mechanism depends on unfolding events in large cytoplasmic regions of the plasma membrane protein that can be recognized by chaperones. These chaperones recruit ubiquitin ligase that catalyses ubiquitination of the unfolded cytoplasmic domain followed by polyubiquitination via the lysine 63 residues of ubiquitin (K63) (Apaja et al., 2013). The polyubiquitin tag then acts as a signal for retrieval into endocytic vesicles and subsequent delivery to the lysosome (vacuole in yeast) in an ESCRT-mediated fashion. This mechanism was described following mammalian studies in which the ubiquitin ligase CHIP is recruited by the chaperones Hsp70 and Hsp90 (Okiyoneda et al., 2010; Apaja et al., 2013). To date, a similar system has not yet been identified in yeast. Although the two ubiquitin ligases Ubr1 and San1 function similarly to mammalian CHIP (Heck, 2010) in the degradation of unfolded transmembrane proteins, these ligases do not seem to play a role in the degradation of unfolded transmembrane proteins. The closest factors to chaperones found to function for plasma membrane quality control are the arrestin-related transport receptors (ARTs) (Lin et al., 2008; Becuwe et al., 2012). They bind to plasma membrane nutrient transporters and help to recruit Rsp5 to the membrane to enhance Rsp5-mediated ubiquitination, hence essentially acting as adaptors (Nikko et al., 2008; Nikko and Pelham, 2009; Zhao et al., 2013). However, the ARTs do not recognize unfolded protein regions and thus do not function homologously to chaperones.

1.4.2. LID-degron quality control mechanism

Fur4 contains in its N-terminus a cytoplasmic 20 amino acid-residue hydrophilic domain, lying between an N-terminal degron and the first transmembrane domain, termed the loop interacting domain (LID). The degron, degradation initiation sequence, is a lysine-containing domain targeted by ubiquitin ligase, Rsp5, for ubiquitin tagging and is known to regulate protein degradation (Keener and Babst, 2013). How Rsp5 is recruited to the plasma membrane is not clear. The LID-degron system has been shown to explain how unfolded Fur4 can be recognized and retrieved in the absence of chaperones (Ravid and Hochstrasser, 2008). In this model, the cytoplasmic LID interacts closely with the intermembrane loop domains through hydrogen bonds causing the degron to be hidden from ubiquitin ligase. This interaction is strongest in the outward facing conformation that is the ground state of the transporter. However, in the presence of stress or excess substrate, the transporter adopts conformational changes that disrupt LID-loop interactions leading to exposure of the degron and consequent ubiquitin-mediated endocytosis. The observation that both substrate-dependent downregulation and quality control are mediated by the LID-degron system differentiates it from chaperone-mediated quality control. This means that conformational changes arising from ordinary transport activity of the nutrient transporter are sufficient to cause its degradation, hence the concept of activity-dependent downregulation. This supports the observation that heat shock can induce the degradation of many membrane proteins, even at temperatures not sufficient to cause unfolding.

The studies done so far on the mechanisms of quality control of transmembrane proteins have been unable to address some pertinent questions. Is accessibility of the

degron sufficient for ubiquitination by Rsp5? Moreover, how do lipid microdomains on the plasma membrane impact ubiquitination reactions? The following chapters will address these questions to improve understanding on the rules governing ubiquitination of transmembrane proteins, especially in the context of lipid microdomains (Grossmann et al., 2008). These chapters will also discuss the use of LOV2 (a light-regulated protein domain) in answering these questions.

Table 1.1 ESCRT complexes and proteins in the MVB pathway (Henne et al., 2010)

ESCRT Protein	Protein Subunits	Function/Role
0		
	Hse1	Binds ubiquitin
	Vps27	Binds PI3P, ubiquitin and recruits Vps27
I		
	Mvb12	Binds ubiquitin
	Vps23	Interacts with Vps27 and binds ubiquitin
	Vps28	Interacts with Vps36
	Vps37	Binds PI3P
II		
	Vps36	Binds PI3P and ubiquitin
	Vps25	Interacts with Vps20
	Vps22	Undefined membrane interactions.
III		
	Vps20	Interacts with Vps4 and 25
	Vps2	Interacts with Vps4
	Vps24	Interacts with Did2
	Snf7	Interacts with Vps4 and form polymerized chains
Vps4 complex		
	Vps4	AAA ATPase mechanoenzyme that remove ESCRT complexes
	Vta1	Promotes ATP activity and consequent Vps4 oligomerization

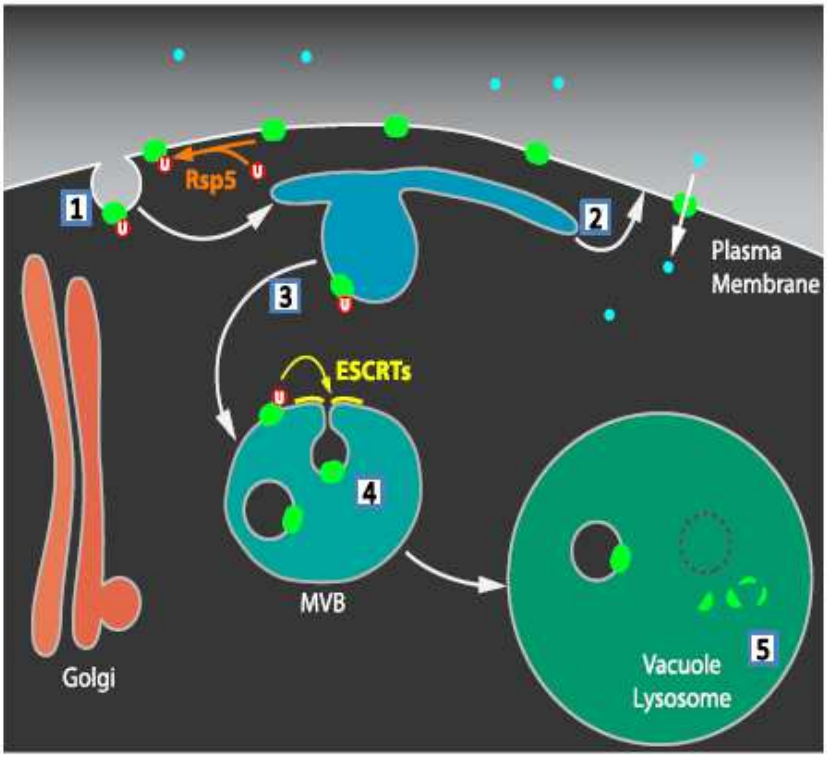

Figure 1.1 Schematic diagram of quality control pathway for transmembrane proteins in yeast. Numbers indicate key steps in quality control. MVB: Multivesicular body, ESCRT: Endosomal complexes required for transport, Rsp5: Ubiquitin ligase.

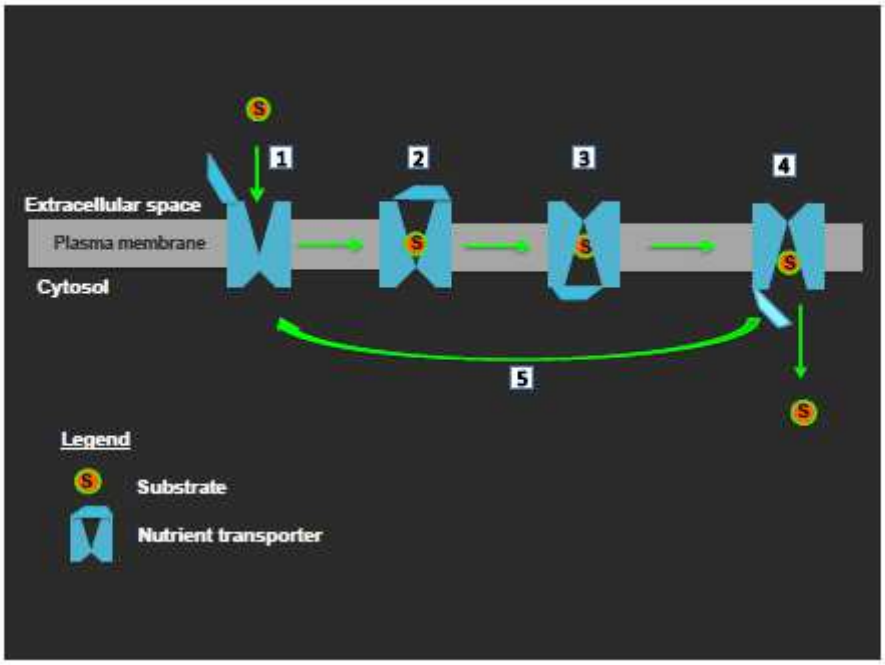

Figure 1.2 Alternating access model for the APC nutrient transporter superfamily.

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CHAPTER 2

INSIGHT INTO UBIQUITINATION IN THE TEMPORAL DOWNREGULATION OF FUR4 AT THE PLASMA MEMBRANE

2.1 Introduction

Ubiquitination is key to the various mechanisms of downregulation described for nutrient transporters. The ubiquitin ligase-Rsp5 is the only known enzyme responsible for the ubiquitination of Fur4 at the plasma membrane. Rsp5 is a soluble protein that is localized to the cytosol. The recruitment of Rsp5 to the plasma membrane is an important regulatory step in the degradation of Fur4. There are three different possible means of Rsp5 recruitment to the degron of nutrient transporters. First, recruitment of Rsp5 could be ART mediated (Figure 2.1). The ARTs might recognize and bind to an exposed degron when the protein unfolds either due to activity, stress or substrate. ART can then recruit Rsp5 to the exposed degron for ubiquitination. This ART-dependent ubiquitination has been proposed to initiate degradation for several nutrient transporters (Liu et al., 2007; Lin et al., 2008; Herrador et al., 2010). However, no ART binding site has been described for Fur4. Second, Rsp5 recruitment could involve a transmembrane adaptor (Figure 2.1). This adaptor will have high affinity for Rsp5 and hence concentrate Rsp5 close to the plasma membrane where it can directly interact with an exposed degron. Third, Rsp5 can be recruited to an exposed degron directly (Figure 2.1).

To investigate if mere accessibility of the ubiquitination site (degron) is sufficient

for Rsp5-dependent ubiquitination, I have used artificial degrons to bypass the additional phosphorylation of PEST-like sequence in the native Fur4 degron. This phosphorylation increases the efficiency of downregulation but is not essential for ubiquitination or degradation (Galan et al., 1996; Marchal et al., 1998; Marchal et al., 2000). Different artificial degrons were tested for their dependence on Rsp5. One of these is the ubiquitination site of carboxypeptidase1 (Cps1). This is a seven amino acid sequence (PVEKAPR) that has been shown to be sufficient for Rsp5-mediated downregulation at the endosome (Katzmann, 2001).

Newly synthesized Fur4 passes through the secretory pathway to reach the plasma membrane. Hence, an artificial degron may be exposed to the quality control system of the ER and Golgi. To solve this problem, the LOV2 domain was utilized. This domain changes its conformation when exposed to blue light that exposes the C-terminal region. Fusing a degron to the C-terminus of LOV2 has been shown in the cytoplasm to function as a light-induced degradation system (Renicke et al., 2013). Therefore, the LOV2-degron system allows for spatial and temporal control of protein ubiquitination.

It is known that Fur4 and other transmembrane proteins are organized into lipid subdomains associated with eisosomes (Malinska et al., 2003; Walther et al., 2006; Simons and Sampaio, 2011; Douglas and Konopka, 2014). Loss of function and downregulation of transmembrane proteins has been reported when this lipid microenvironment is perturbed (Dupre et al., 2003; Bultynck, 2006), highlighting the importance of maintaining the proteins in the proper lipid milieu (Lauwers et al., 2007; Pineau et al., 2008; Payet et al., 2013). Interestingly, endocytosis of Fur4 does not occur within eisosomes, indicating that Fur4 has to move out of these lipid domains in order to

be degraded. How Fur4 moves between eisosomes and the sites of endocytosis is unknown. Furthermore, it is not clear in which lipid domain the ubiquitination of Fur4 occurs.

The present study represents the first attempt to determine the links between eisosomes and plasma membrane protein quality control.

2.2 Materials and methods

2.2.1 Strains and growth conditions

Plasmids were amplified in the *Escherichia coli* host XL1-blue (Inoue et al., 1990). The bacterial strains were grown at 37ºC in LB medium (1% tryptone, 0.5% yeast extract, 1% sodium chloride) supplemented with 100g/ml ampicillin for a selection of transformants. *S.cerevisiae* strains used in this study are listed in Table 2.1. Yeast cells were grown in YPD (1% yeast extract, 2% peptone, and 2% glucose) or in a synthetic minimal medium (SD; 0.67% yeast nitrogen base without amino acids [Difco; BD] and 2% glucose supplemented with essential amino acids) (Sherman et al., 1979). All Fur4clonings were based on the plasmid pJK17 [p (*SNF7*)-FUR4-GFP]. In yeast, GFP-Fur4 constructs were expressed from the constitutive *SNF7* promoter and analysis was performed using cells grown to mid-log phase.

2.2.2 Light-induced degradation

For light-induced degradation experiments, I used a commercially available light source (verilux happy lite mini plus 26W output. Model: VT01-SB). I used a lamp that emits the full spectrum of white light knowing that the LOV2 domain only absorbs in the blue light range (460-470nm). Cells were grown in transparent standard laboratory glass flasks containing minimal media lacking uracil at 30ºC. These flasks were placed

approximately 5cm from the light source. The culture flasks containing cells without illumination were wrapped in aluminium foil and placed within the same incubator to ensure uniform growth conditions. There was no additional effort made to protect cells from ambient light in the growth phase. Wild type yeast cells expressing GFP-*ΔN60*Fur4 and GFP-*ΔN60*Fur4-LOV2-Cps1 were grown to OD_{600}=0.5. Growth is then continued for additional 60 minutes to OD_{600}=0.6 with or without illumination. In the set of experiments involving uracil uptake, the same set-up was used whereby cells were exposed to both light and uracil (5mg/L), either light or uracil and neither. In both cases, following the experiments, cells were prepared for microscopy.

2.2.3 Fluorescence microscopy

Cells were grown to OD_{600}=0.6 and analysed by fluorescence microscopy using a deconvolution microscope (DeltaVision; Applied Precision). Images were quantitated using PHOTOSHOP software. I photographed and deconvolved images of 50 random cells and saved them as a projection in PHOTOSHOP format. Images were analysed for endocytic activity (early endosome, ILV or vacuolar signal) relative to plasma membrane GFP-Fur4 signal. Data were recorded and analyzed in excel.

2.3 Results

2.3.1 Degron exposure is insufficient for Rsp5-mediated ubiquitination in Fur4 endocytosis

The ubiquitination site (called degron) in the N-terminus of Fur4, which occupies the first 60 amino acid-residues, is known to be essential in the Rsp5-dependent endocytosis of Fur4 (Marchal et al., 2002). Degron exposure was later proven to be consequent to conformational changes in the transporter that are transmitted to the LID domain (Keener and Babst, 2013). It has also been shown that

deleting this degron prevents endocytosis (Keener and Babst, 2013). Direct recruitment of Rsp5 by the exposed Fur4 degron is one of the proposed mechanisms of targeting the soluble Rsp5 to the site of ubiquitination.

To test if exposure of the degron is sufficient to trigger ubiquitination and subsequent downregulation of Fur4, I separated the conformation sensing function of the LID domain from degron exposure by deleting the native degron and replacing it with the light-regulated LOV2-degron (GFP-*ΔN60*Fur4-LOV2-degron (CPS1)). This construct was expressed in yeast cells and the trafficking of the GFP-tagged fusion protein was monitored before and after 1 hour of illumination with light (to induce the LOV2-degron). Surprisingly, GFP-*ΔN60*Fur4-LOV2-degron remained stably localized to the plasma membrane with no evidence of endocytic activity (early endosome, MVB or vacuolar signal) (Figure 2.3). This result indicated that even after a light induced degron exposure in an Rsp5-competent cell, there was no recruitment of Rsp5 to the degron. Assuming that the LOV2-degron system is functional at the plasma membrane, the result suggested that degron exposure is not sufficient to trigger Fur4 endocytosis. One possible explanation is that Rsp5 is not directly recruited to the degron but requires an adaptor protein. Our subsequent results show that this putative adaptor recognized the artificial degron and mediated Rsp5-dependent endocytosis.

2.3.2 Ubiquitination and endocytosis of Fur4 occur after lateral translocation

Previous studies have shown that plasma membrane proteins in *S. cerevisiae* can be divided into three groups based on their spatial distribution at the cell surface: discrete patches, mesh-shaped compartments interspersed between the patches, or

homogeneous distribution (Malinska et al., 2003; Grossman et al., 2006**)**. Fur4 has been shown to be localized to discrete patches, called eisosomes, alongside transmembrane proteins like Sur7, whose function is currently undefined (Young et al., 2002). It has also been shown that Fur4 is more readily endocytosed when it is dissociated from the eisosomes (Dupre et al., 2003; Bultynck, 2006). However, where the ubiquitination reactions occur is yet to be shown. This information can be crucial to understand Rsp5 recruitment and function in Fur4 ubiquitination.

To test if ubiquitination of an exposed degron of Fur4 occur after lateral translocation to the mesh-like more fluid membrane compartment, I first tested the colocalization of Fur4 with the eisosome marker Sur7. Similar to previous studies (Dupre et al., 2003; Bultynck, 2006), I found that in the presence of uracil, Fur4(ΔN60)-mCherry dissociated from the eisosomes and translocated laterally to the more fluid mesh-like compartment (Figure 2.4). This observation suggested that binding of the substrate caused Fur4 to move out of the eisosome compartment by a mechanism that does not require ubiquitination.

I then expressed GFP-*ΔN60*Fur4-LOV2-degron(Cps1) in yeast and monitored the trafficking of this fusion protein in the presence or absence of 1h-illumination and/or uracil. I observed rapid endocytosis of the Fur4 construct only in the cells exposed to both light and uracil. As expected, the control fusion protein GFP-*ΔN60*Fur4 was not downregulated (Figure 2.5). Together the results suggested that exposure of the degron is essential but insufficient, as uracil-induced conformational changes are also independently required.

2.4 Discussion

Fur4 is a transmembrane protein that functions as a high affinity uracil permease in yeast. It belongs to the nucleobase:cation symporter-1 (NCS1) family of transporters (Jund et al., 1988). The quality control of Fur4 is dependent on its endocytosis from the plasma membrane and trafficking through the MVB pathway to the vacuole for degradation. Fur4 and other nutrient transporters have been shown to localize at the plasma membrane to eisosomes (Walther et al., 2006). Eisosomes refer to the complex of proteins peripherally associated with the cytosolic faces of the plasma membrane microdomains that are organized into discrete patches (Reviewed in Douglas and Konopka, 2014). These discrete patches have been described as membrane compartment of Can1 (MCC). Nutrient transporters are known to move laterally out of eisosomes before being endocytosed (Lauwers et al., 2007; Pineau et al., 2008; Payet et al., 2013). Endocytosis is a key event in the control of Fur4 function and it is regulated by an Rsp5-mediated ubiquitination reaction. How the soluble Rsp5 protein is targeted to the degron of Fur4, and where this ubiquitination reaction occurs remain open questions in the field. In addition, the role of eisosomes in regulating Fur4 turnover is not clear.

To answer these questions, I used a LOV2-based photosystem to separate conformational changes in Fur4 caused by substrate binding from degron exposure. Our results suggested that the simple exposure of a degron is insufficient for Fur4 endocytosis. When cells expressing GFP-*ΔN60*Fur4-LOV2-degron(Cps1) were illuminated for 60 minutes and analysed by fluorescence microscopy, no Fur4 endocytosis was observed. One possible explanation for this result is that eisosome-localized Fur4 is not accessible by Rsp5 (Figure 2.3). This model was supported by the

observation that triggering the lateral translocation of Fur4 out of eisosomes to the mesh-like membrane compartment allowed for light-induced endocytosis. Growing GFP-*ΔN60*Fur4-LOV2-degron(Cps1) expressing cells in the presence of both uracil, which caused Fur4 to move out of the eisosome (Figure 2.4), and light triggered Fur4 endocytosis (Figure 2.5, column 4).

Taken together, these results suggested that substrate binding, which causes Fur4 to move out of eisosomes, is a prerequisite for ubiquitination. A previous study has shown that endocytosis of Fur4 is unaffected in ART mutants (Keener and Babst, 2013); hence, ART-mediated recruitment of Rsp5 is not likely in case of Fur4. Therefore, I propose that Rsp5 localizes to the fluid, mesh-like membrane by binding to a yet unidentified transmembrane adaptor (TA). This plasma membrane pool of Rsp5 is then able to ubiquitinate substrate-bound Fur4 that has moved out of eisosomes and exposes a degron (Figure 2.7).

The mechanism of Fur4 translocation out of eisosomes could possibly be explained by intrinsic substrate-induced conformational changes. It is known that proteins are maintained within eisosomes partly by having long transmembrane domains which anchor the protein in the thicker membrane structures of the raft-like eisosomes (Kutti and Henderson, 2010). Based on structural analyses of the bacterial homologue Mhp1, I predict that substrate-induced conformational changes in Fur4 could cause a kinking of the first two anchoring helices allowing lateral translocation from the eisosomes to the more fluid mesh-like membrane compartment. Here, the transmembrane adaptor-localized Rsp5 can then catalyze the ubiquitination event that will trigger Fur4 endocytosis (Figure 2.7).

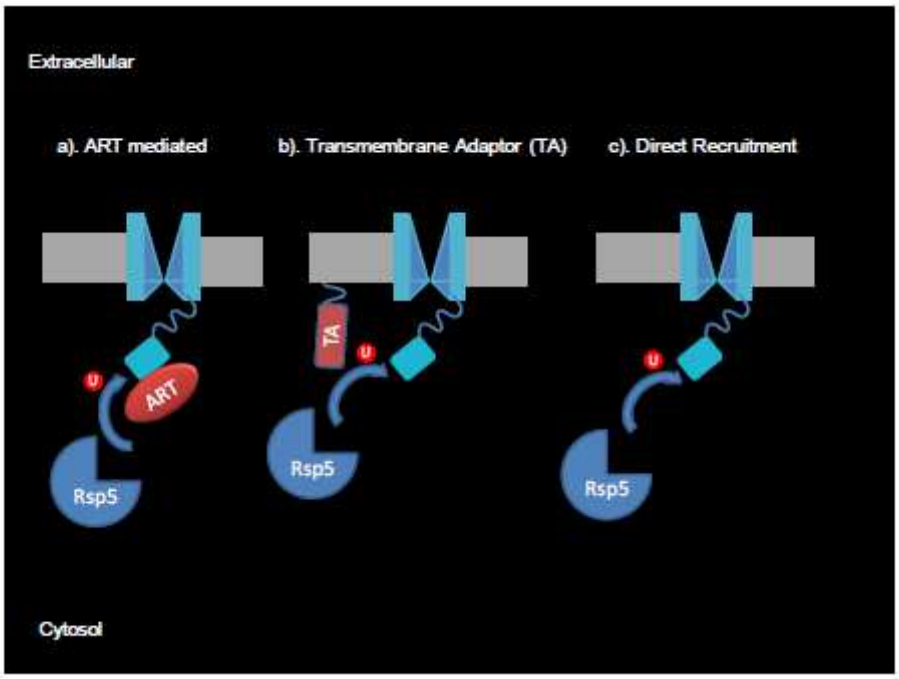

Figure 2.1 Possible mechanisms of Rsp5 recruitment to exposed degron.

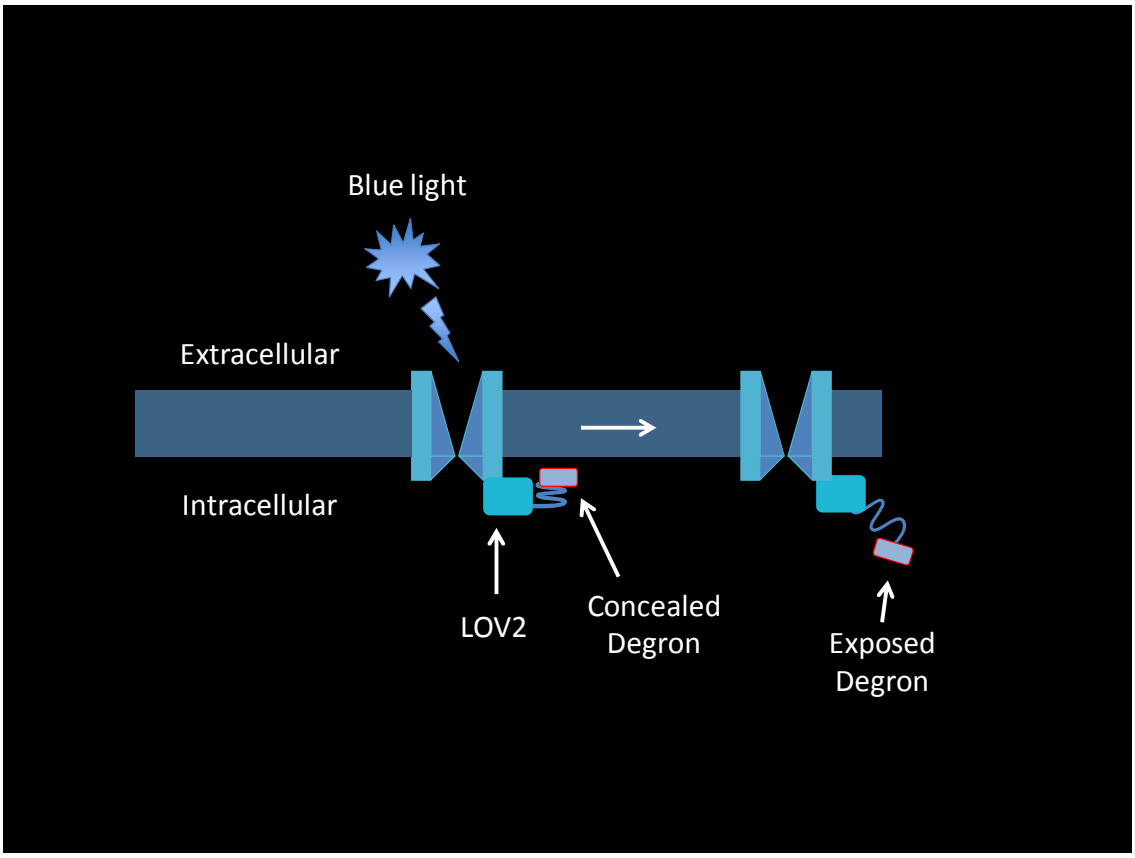

Figure 2.2 The LOV2 photosytem.

Table 2.1 Strains and plasmids used in this study

Strain or Plasmid	Descriptive name	Genotype or description	Source
Strain			
SEY6210	WT	MAT leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801suc2-Δ9	(Robinson et al., 1979)
Plasmids			
pMA1	P(*PRC1*)-FUR4 -GFP-LOV2	URA3(pRS416) P(*PRC1*)-FUR4 (Δ60)-GFP-LOV2	This study
pMA2	P(*PRC1*)-FUR4 -GFP-LOV2	URA3(pRS426) P(*PRC1*)-FUR4 (Δ60)-GFP-LOV2	This study
pMA3	P(*PRC1*)-FUR4 -GFP-LOV2-CPS	URA3(pRS416) P(*PRC1*)-FUR4(Δ60) -GFP-LOV2-PVEKAPR	This study
pMA4	P(*PRC1*)-FUR4 -GFP-LOV2-CPS	URA3(pRS416) P(*PRC1*)-FUR4(Δ60) -GFP-LOV2--PVEKAPR	This study
pJK17	P(*PRC1*)-FUR4 (Δ60)-GFP	URA3(pRS416) P(*PRC*1) - FUR4(Δ60)-GFP	(Keener et al., 2013)

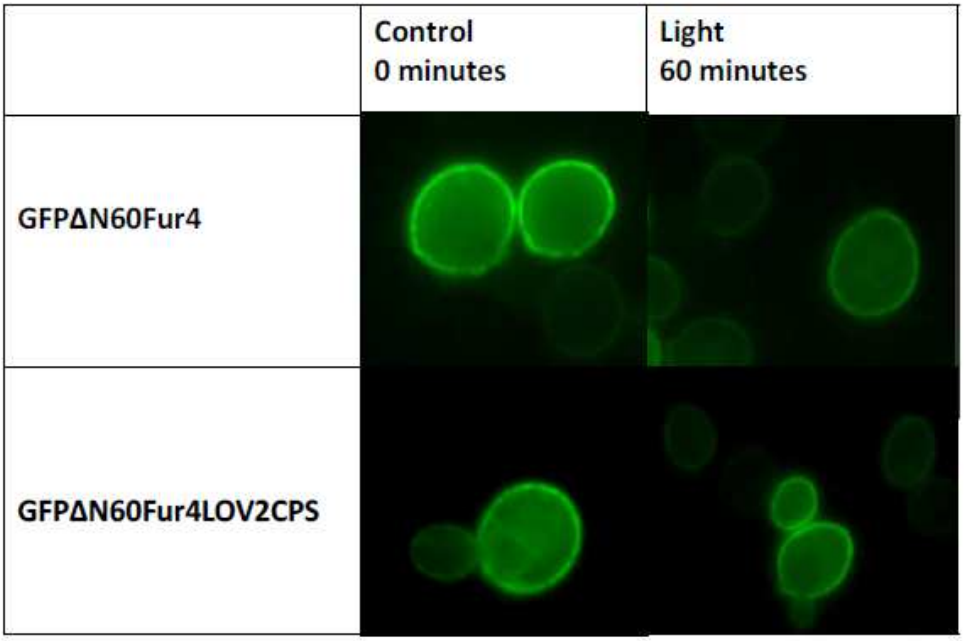

Figure 2.3 Degron exposure is insufficient for Fur4 endocytosis**.** The downregulation of GFP-*ΔN60*Fur4 or GFP-*ΔN60*Fur4-LOV2-degron(Cps1) in yeast cells grown in the absence of light (first column) and presence of light (second column) was analysed by fluorescence microscopy. Light-induced LOV2-degron exposure is insufficient for downregulation of Fur4.

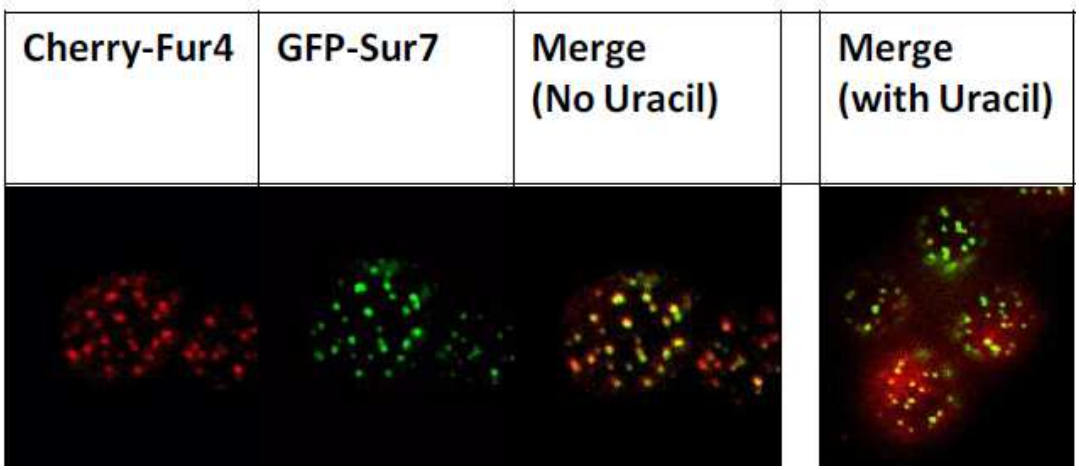

Figure 2.4 Fur4(ΔN60)-mCherry dissociates from Sur7-GFP in presence of uracil. Surface views of yeast cells expressing Fur4(ΔN60)-mCherry and Sur7-GFP before (columns one to three) and 30 minutes after the addition of 5mg/L uracil to the medium (fourth column) are show. ΔN60Fur4 translocation from eisosome-localized Sur7 to the lateral MCP suggests that Fur4 ubiquitination and endocytosis likely occur in the MCP

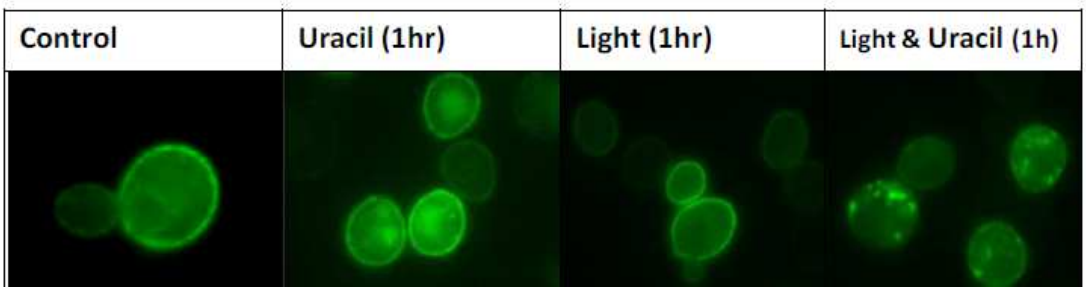

Figure 2.5 Ubiquitination and endocytosis of Fur4 occurs after lateral translocation. Downregulation of GFP-*ΔN60*Fur4-LOV2-degron(Cps1) in the presence of either uracil, light or both uracil and light together. Yeast cells used for the experiment were transformed with a plasmid expressing GFP-*ΔN60*Fur4-LOV2-degron(Cps1) and were analysed by fluorescence microscopy. Endocytosis of Fur4 requires both degron exposure and substrate-induced conformational changes.

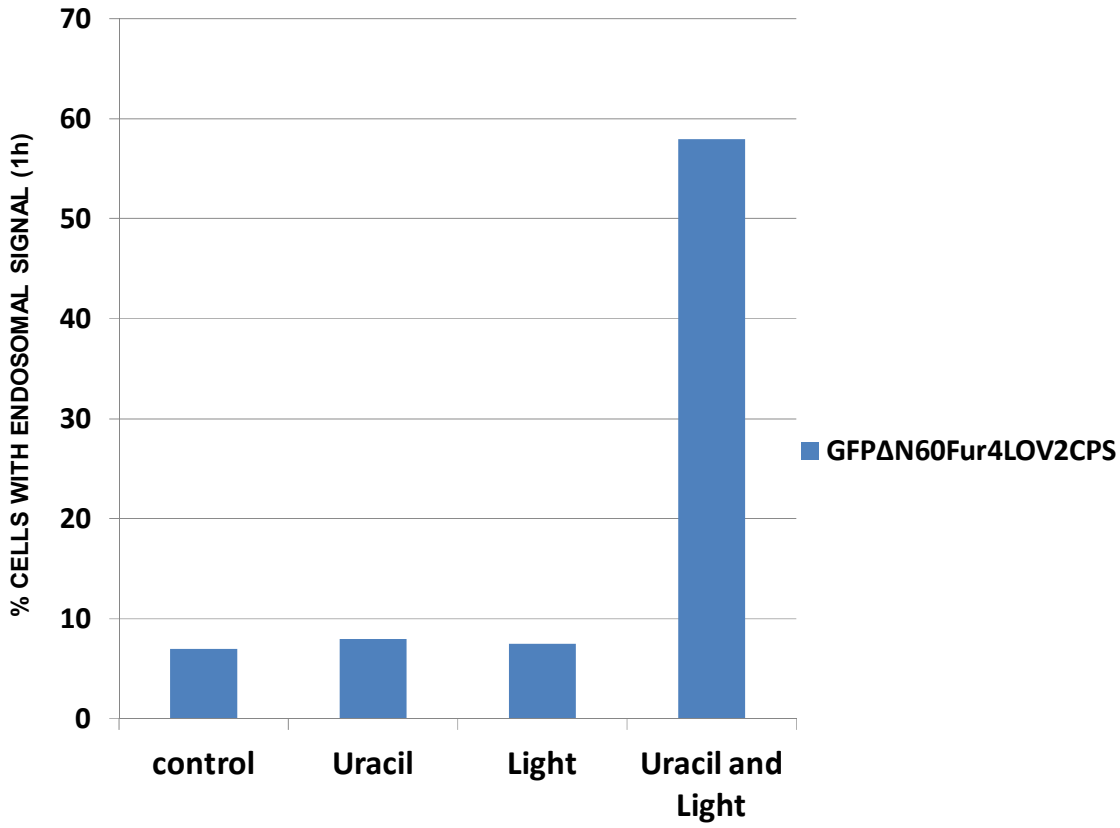

Figure 2.6 Quantitation of the fluorescence microscopy shown in figure 2.5. The graph shows the percentage of cells with obvious endosomal structures. 50 cells were quantified for each experiment. This indicates that light-induced degron exposed and uracil-induced conformational changes are required for the translocation of Fur4 out of the eisosomes to the lateral fluid and mesh-like MCP.

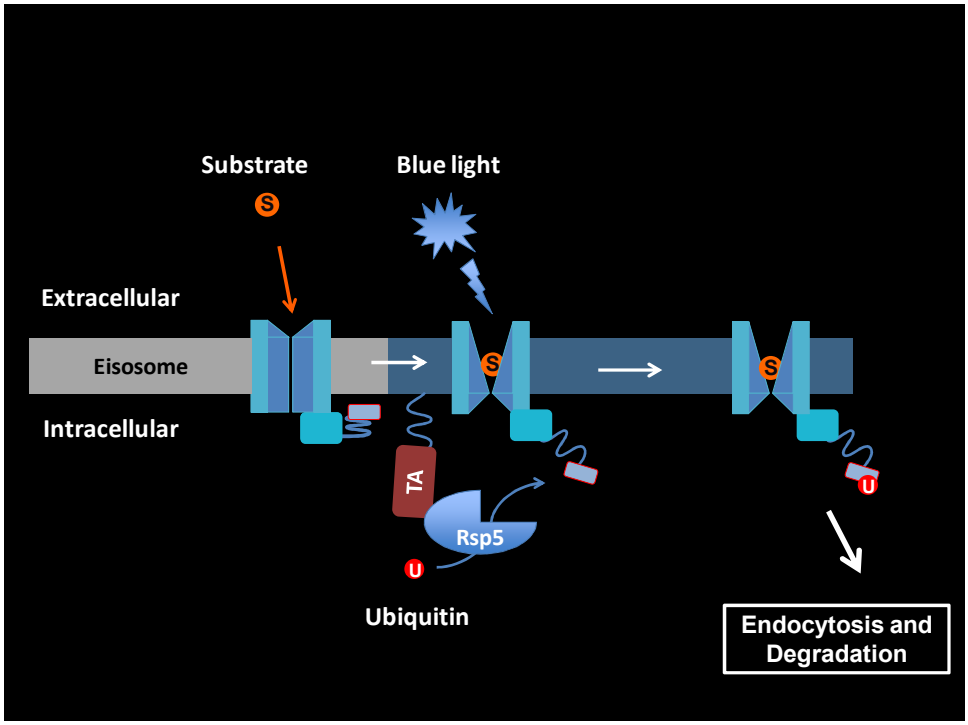

Figure 2.7 Model of transmembrane-adaptor (TA)-mediated Rsp5 recruitment in Fur4 endocytosis.

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CHAPTER 3

CONCLUSION

3.1 Concluding remarks and future perspectives

Protein homeostasis is crucial for normal cellular functioning. Quality control of transmembrane proteins upholds cellular integrity and activity-dependent turnover aids in the regulation of overall nutrient transporter quantity on the plasma membrane. These regulatory systems ensure a proper balance between cellular nutrient needs and environmental conditions.

The primary question that this work has started to answer is: What is the effect of the eisosome domain on nutrient transporter turnover. Current opinion in the field is that the nutrient transporter Fur4 resides within the eisosome domain where it actively pumps uracil. In this project, I found that as soon as uracil binds, Fur4 moves out of the eisosomes in an ubiquitination-independent fashion (Figure 2.4 in Chapter 2). This suggests that Fur4 translocation is likely driven by conformational changes. In the same vein, Fur4 likely prefers to stay in the eisosomes in the ground state, since it moves out of eisosomes only in the presence of uracil, a substrate that causes a switch from ground state to the outward facing occluded conformation. Fur4 translocates laterally to the mesh-like fluid microcompartment of Pma1 (MCP) where yet unidentified transmembrane adaptors likely aid in Rsp5 targeting to the highly active Fur4 protein (Figure 2.7 in Chapter 2). A direct recruitment of Rsp5 to the exposed degron of Fur4 is

not likely because Fur4 endocytosis was only observed in presence of uracil, suggesting that localization of Fur4 to the MCP is essential for ubiquitination to occur. In support of this model, the ART-mediated recruitment of Rsp5 has been disputed by recent data that show endocytosis of Fur4 in ART-deleted yeast strains (Keener and Babst, 2013).

Results from this work also suggest a further role of eisosomes in the regulation of global cellular plasma membrane nutrient transporter expression. I propose that eisosomes serves as a storage site for inactive Fur4. This allows the cell to control the overall quantity of nutrient transporter on its surface. In the fluid mesh-like MCP, the proton pump Pma1 is responsible for building the proton gradient that drives proton-dependent Fur4 transporter activity. Generating this proton gradient is energy-dependent; hence, the cell must regulate the overall amount of nutrient transporter present at the cell surface at a given point in time. Our data suggest that storage of inactive ground state Fur4 eisosomes can be an energy conserving mechanism in the cell.

The presented study has furthered our understanding of nutrient transporter quality control and turnover. However, our model makes several predictions that require further studies. For example, the identity of the plasma membrane adaptor for Rsp5 is unknown. Several Rsp5 adaptors have been identified that function at the ER or the endosomal system. However, it is not clear if any of these adaptors are also acting at the cell surface. It has been shown that proteins are retained within eisosomes partly by having long transmembrane domains which anchor the protein in the bulky membrane structures of the raft-like eisosomes (Kutti and Henderson, 2010). How are mutations in these transmembrane domains of Fur4 affecting its localization to the eisosome?

The work presented here has laid the groundwork for future exploration of plasma

membrane protein downregulation and turnover.

3.2 References

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